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Influence of nitrite accumulation on "*Candidatus* Accumulibacter" population structure and enhanced biological phosphorus removal from municipal wastewater

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HIGHLIGHTS

- Operational data of 369 days were from MUCT process treating municipal wastewater.
- Relevance of population dynamics of clade-level Accumulibacter to EBPR was studied.
- Largest Clade IID was associated with good performance of EBPR under nitritation.
- Clade IID as dominant Accumulibacter performed denitrifying P removal via nitrite.
- Clade I of 5% existed under nitrification, and then disappeared under nitritation.

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ABSTRACT

A modified University of Cape Town (MUCT) process was used to treat real municipal wastewater with low carbon to nitrogen ratio (C/N). To our knowledge, this is the first study where the influence of nitrite accumulation on "*Candidatus* Accumulibacter" clade-level population structure was investigated during nitritation establishment and destruction. Real time quantitative PCR assays were conducted using the polyphosphate kinase 1 gene (*ppk*1) as a genetic marker. Abundances of total "*Candidatus* Accumulibacter", the relative distributions and population structure of the five "*Candidatus* Accumulibacter" clades were characterized. Under complete nitrification, clade I using nitrate as electron acceptor was below 5% of total "*Candidatus* Accumulibacter". When the reactor was transformed into nitritation, clade I gradually disappeared. Clade IID using nitrite as electron acceptor for denitrifying phosphorus (P) removal was always the dominant "*Candidatus* Accumulibacter" throughout the operational period. This clade was above 90% on average in total "*Candidatus* Accumulibacter", even up to nearly 100%, which was associated with good performance of denitrifying P removal via nitrite pathway. The nitrite concentrations affected the abundance of clade IID. The P removal was mainly completed by anoxic P uptake of about 88%. The P removal efficiency clearly had a positive correlation with the nitrite accumulation ratio. Under nitritation, the P removal efficiency was 30% higher than that under complete nitrification, suggesting that nitrite was appropriate as electron acceptor for denitrifying P removal when treating carbon-limited wastewater.

1. Introduction

Enhanced biological phosphorus removal (EBPR) as an economic and efficient means has been widely applied in full-scale wastewater treatment plants (WWTPs). For the treatment of municipal wastewater, lack of organic carbon source in raw wastewater is becoming the limiting factor for biological nutrients removal. Denitrifying phosphorus removal implies that polyphosphate-accumulating organisms (PAOs) using nitrate or nitrite as electron acceptors instead of oxygen for poly-β-hydroxyalkanoates (PHAs) oxidation under anoxic conditions. It has been intensively studied due to incorporating both nitrogen and phosphorus (P) removal (Kuba et al., 1997; Carvalho et al., 2007; Zafiriadis et al., 2011). Short cut nitrification-denitrification is defined that

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ammonia is oxidized to nitrite (nitritation), and then directly reduced to nitrogen gas (denitritation) (Hellinga et al., 1998; Zhu et al., 2008). Compared with the traditional biological nutrients removal, denitrifying P removal and nitritation/denitritation leads to a considerable saving in energy cost and carbon source. If nitrite generated from nitritation is used as electron acceptor for denitrifying P removal via nitrite pathway, carbon sources and aeration costs would be further reduced. Meanwhile, nitrite inhibition would also be eliminated. That is favorable for the treatment of carbon-limited wastewater. Therefore, studies on population structure of PAOs and the ability of PAOs to reduce nitrite are significant to achieve denitrifying P removal via nitrite pathway.

At present, although PAOs employed in EBPR have not been cultivated in isolation, culture-independent approaches identified PAOs primarily affiliated with the *Rhodocyclus* group in the *Betaproteobacteria*, and were named "*Candidatus* Accumulibacter phosphatis" (Crocetti et al., 2000). Surveys from full-scale WWTPs and laboratory-scale reactors confirmed "*Candidatus* Accumulibacter" are the dominant PAOs (Saunders et al., 2003; He et al., 2007; Peterson et al., 2008; He and McMahon, 2011a). Extensive research has been carried out to investigate "*Candidatus* Accumulibacter" microbiology and ecophysiology with molecular tools. However, disagreements evident in these studies, such as carbon metabolic pathway and the capability of "*Candidatus* Accumulibacter" to reduce nitrate, indicated "*Candidatus* Accumulibacter" diversity in phenotype and genotype (Oehmen et al., 2007; He and McMahon, 2011a). The 16S rRNA gene is the most commonly used phylogenetic marker to investigate "*Candidatus* Accumulibacter" phylogeny. Yet it cannot reveal fine-scale differences within "*Candidatus* Accumulibacter" since 16S rRNA is highly conservative (He and McMahon, 2011a). Polyphosphate [poly(P)] kinase 1 (PPK1) is the key enzyme to catalyze intracellular synthesis of poly(P), which affects PAOs metabolic activity and performance of EBPR. The *ppk*1 gene encodes PPK1, which is a single-copy gene in "*Candidatus* Accumulibacter" and evolves four times faster than 16S rRNA. And thus it is a good genetic marker to reveal fine-scale population structure (Martin et al., 2006; Kunin et al., 2008; He and McMahon, 2011a).

Based on *ppk*1 genes, "*Candidatus* Accumulibacter" is composed of two types (I and II, respectively), each type consisting of a number of clades (Clade IA, IB, IC, ID, IE, IIA, IIB, IIC, IID, IIE, IIF, IIG) (Peterson et al., 2008; He and McMahon, 2011a). Previous studies based on *ppk*1-specific PCR primers and probes showed that the enriched "*Candidatus* Accumulibacter" by synthetic wastewater was mainly phylogenetically related to clade IA and IIA (He et al., 2010; He and McMahon, 2011b; Graciela and Christof, 2011), and the "*Candidatus* Accumulibacter" from WWTPs was primarily affiliated with type II (McMahon et al., 2007; He et al., 2008). Generally, lab-scale reactors with synthetic wastewater showed a lower "*Candidatus* Accumulibacter" diversity than full-scale systems (He and McMahon, 2011a). Studies regarding denitrifying P removal mainly focused on the capabilities of "*Candidatus* Accumulibacter" to utilize different types of electron acceptors, i.e. oxygen, nitrite and nitrate (Carvalho et al., 2007; Flowers et al., 2009; Lanham et al., 2011; Zafiriadis et al., 2011), and operational control of denitrifying P removal process (Wang et al., 2011), especially on nitrate reduction (Kim et al., 2013).Flowers et al. (2009) found that clade IA-enriched sludge could couple nitrate reduction with P-uptake, but clade IIA could not. Regarding the effect of nitrite, most of researches were related to nitrite inhibition on aerobic/anoxic P-uptake of "*Candidatus* Accumulibacter" (Zhou et al., 2010, 2012). Very limited studies were conducted on the nitrite reduction of clade-level "*Candidatus* Accumulibacter".

Studies on "*Candidatus* Accumulibacter" using *ppk*1 gene had the following characteristics. Firstly, most of studies were about the "*Candidatus* Accumulibacter"-enriched culture using simply synthetic wastewater (Kim et al., 2010; Lanham et al., 2011; Kim et al., 2013). The population structure of "*Candidatus* Accumulibacter" in synthetic wastewater was relatively simpler than that in real wastewater. Secondly, although surveys of "*Candidatus* Accumulibacter" in full-scale EBPR plants were extensively carried out, sludge samples were taken at certain operational condition. Due to the restrictions resulted from operation and management of WWTPs, influencing factors can not be optionally changed to gain a good insight into "*Candidatus* Accumulibacter" dynamics. The processes incorporating both nitrogen and P removal is commonly required in WWTPs, which is more complicated in microbial communities and interactions than a sole EBPR system. Therefore, what dominates the observed dynamics of "*Candidatus* Accumulibacter" population structure is largely unclear. Lastly, regarding studies of "*Candidatus* Accumulibacter" denitrifying ability, nitrite or nitrate as electron acceptor was externally added into a reactor at the beginning of anoxic phase rather than generated from nitrification process, which was different from real wastewater treatment system. These characteristics above mentioned may restrict a generalization of knowledge obtained from previous studies to real wastewater treatment systems. However, very limited studies were carried out regarding the dynamics of "*Candidatus* Accumulibacter" population structure in the real wastewater systems with complex influent composition, dynamic operating conditions and multiple biochemical processes (Slater et al., 2010; Albertsen et al., 2012). Especially, to our knowledge, no study was reported regarding the relevance of population dynamics of different "*Candidatus* Accumulibacter" clades to the performance of denitrifying P removal via nitrite pathway in a real municipal wastewater system.

In a system coupling denitrifying P removal with nitritation, the dynamics of types and concentrations of electron acceptor (nitrite or nitrate) resulted from the transition between nitritation and nitrification will affect population structure of "*Candidatus* Accumulibacter" and performance of EBPR. Therefore, this study aims to (1) investigate the relevance of clade-level population dynamics of "*Candidatus* Accumulibacter" to the performance of EBPR in a real municipal wastewater system, (2) reveal the mechanism of influence of nitrite accumulation on "*Candidatus* Accumulibacter" population structure and EBPR, and (3) achieve denitrifying P removal via nitrite pathway from real municipal wastewater based on the optimization of clade-level "*Candidatus* Accumulibacter" and operational conditions.

2. Materials and methods

2.1. Experimental reactor and operation

Experimental system consisting of a MUCT reactor of 70 L and a secondary settler of 24 L is shown in Fig. S1 in the supplementary data. The MUCT reactor was divided into seven chambers. The first four chambers with mechanical mixers were used as anaerobic or anoxic zones and the following three with air diffusers were used as aerobic zones. The first chamber provided an anaerobic zone for P-release and for influent. The second chamber was anoxic zone I for denitrification of returned sludge from secondary settler with a recycle ratio of R_1. The recycle ratio of mixed liquid from anoxic zone I to anaerobic zone was R_2. The third and fourth chambers were anoxic zone II for denitrification of recycled nitrification liquid from the last aerobic chamber with a recycle ratio of R_3.

2.2. Wastewater and seed sludge

Municipal wastewater from a campus sewer line was pumped into a storing tank for sedimentation, and then fed into the reactor. Table S1 in the supplementary dada shows the influent

characteristics. The average C/N ratio was about 3.0, and thus the organic carbon source in raw wastewater was typically limiting. Seed sludge was taken from a municipal wastewater treatment plant using an anaerobic-anoxic-aerobic process in Beijing. Nitrification/denitrification and phosphorus removal was performed well in this WWTP without nitrite accumulation. The composition of municipal wastewater treated in this WWTP is similar with that used in this study.

2.3. Experimental procedure

The experimental data of this study were collected from day 43 to day 411 of the MUCT operational period (total 369 d) including 11 successive phases. Operational conditions throughout the experimental period are shown in Table 1. During the phases I–V, the temperature of wastewater and reactor was maintained at ambient temperature. During phases VI-XI, the reactor temperature was controlled at 28 ± 2 °C by electronic heater and temperature sensor. The 11 successive phases were divided according to operating parameters, such as hydraulic retention time (HRT), sludge recycle ratio (R_1), anoxic recycle ratio (R_2) and nitrification liquid recycle ratio (R_3). Changing these parameters aimed to achieve nitritation and high nitrite accumulation. Phases I, II and VI aimed to investigate the effect of HRT, DO concentration and wastewater temperature on establishment of nitritation. Phases III, VII, VIII, IX and X were in stable performance of nitritation. Phases IV, V and XI were nitritation-destroyed period due to low wastewater temperature and high DO concentration. Population structure of clade-level "*Candidatus* Accumulibacter" and EBPR performance was investigated during the phases I-XI.

2.4. Analytical methods

Phosphate (PO_4^{3-}-P), chemical oxygen demand (COD), ammonia (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N), mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) was measured according to the APHA standard methods (APHA, 1998). Volatile fat acids (VFAs) were measured using Agilent 6890N gas chromatography (GC) with an Agilent DB-WAXetr column (30 m × 1.0 μm × 0.53 mm). PHAs analysis was conducted using Agilent 6890N GC with an Agilent DB-1 column. DO concentrations were measured on-line using DO meters (Mutil 340i, WTW, Germany). Nitrite accumulation ratio (NAR) was calculated according to the following formula 1:

$$NAR = \frac{C(NO_2^- - N)}{C(NO_2^- - N) + C(NO_3^- - N)} \quad (1)$$

where $C(NO_2^-$-N) and $C(NO_3^-$-N) is NO_2^--N and NO_3^--N concentration in effluent of the last aerobic zone, respectively.

Fluorescence in-situ hybridization (FISH) was used to quantify the relative abundance of PAOs to total eubacteria (Olympus BX61 fluorescence microscope with Image-Pro Plus 6.0 software). The 16S rRNA-targeted oligonucleotide probes for FISH analysis were EUB_{mix} (an equimolar mixture of probes EUB338, EUB338-II and EUB338-III) target for eubacteria and PAO_{mix} (an equimolar mixture of probes PAO462, PAO651 and PAO846) target for PAOs (Crocetti et al., 2000).

2.5. Real-time quantitative PCR

The standard curves were established for quantification of *ppk*1 genes of five "*Candidatus* Accumulibacter" clades in sludge samples throughout the operational period. The sludge samples were collected from the last chamber of aerobic zone on day 43, 82, 119, 131, 160, 234, 255, 302, and 339. The oligonucleotide sequences of the clade-specific "*Candidatus* Accumulibacter" *ppk*1 primers, "*Candidatus* Accumulibacter" and bacterial 16S rRNA primers are shown in Table S2 in the supplementary data.

PCR reaction mixture with a total volume of 25 μl contained 1 μl DNA template, 12.5 μl GoTaq Green Master Mix (2-fold) (Promega GoTaq Green Master Mix, USA), 1 μl forward primer (10 mM), 1 μl of reverse primer (10 mM), and 9.5 μl of nuclease-free water. The PCR program used for "*Candidatus* Accumulibacter" *ppk*1 genes, "*Candidatus* Accumulibacter" and bacterial 16S rRNA genes was as follows: 10 min at 95 °C, followed by 25–35 cycles of 40 s at 95 °C, 60 s at T °C and 2 min at 72 °C, and a final cycle consisting of 5 min at 72 °C. The annealing temperature (T °C) of different "*Candidatus* Accumulibacter" clade is given in Table S2. The plasmid in the positive clones was extracted and purified using MiniBEST plasmid purification Kit (Ver.3.0, TaKaRa, Japan), and the concentration was measured with a spectrophotometer (NanoDrop ND-1000, Thermo, USA). Copy numbers were calculated based on mass concentration and average molecular weight of the plasmid. Ten-fold serial dilutions of known copy number were used as standard DNA. Quantitative PCR mixture was prepared in a total volume of 25 μl using Brilliant II SYBR Green QPCR Master Mix kit (Agilent Technologies, USA). The quantitative PCR program except Acc-IID consisted of 3 min at 95 °C, 30–55 cycles of 30 s at 94 °C and 45 s at T °C, and a final cycle of 30 s at 72 °C using a QPCR instrument (Mx3005P, Agilent Technologies, USA). The annealing temperature (T °C) is given in Table S2. In order to eliminate the impact of primer dimer on clade IID quantification, a 5 s holding at 84 °C was added after extension step in this study. The temperature of this step (84 °C) was higher than melting temperature of the primer dimer, but lower than the target product.

Table 1
Experimental scheme of MUCT process treating municipal wastewater.

Phase	Influent flow rate ($L \cdot h^{-1}$)	HRT (h)	Sludge recycle ratio R_1 (%)	Anoxic recycle ratio R_2 (%)	Nitrification liquid recycle ratio R_3 (%)	T (°C)	DO ($mg \cdot L^{-1}$)	SRT (d)
I(43–57d)	6.67	10.5	100	100	300	23–28	0.5	25 ± 5
II(58–86d)	8.75	8	100	120	300	23–28	0.5	25 ± 5
III(87–121d)	8.75	8	80	120	300	23–28	0.5	25 ± 5
IV(122–151d)	11.67	6	80	120	300	18–23	0.5	25 ± 5
V(152–180d)	8.75	8	80	120	300	18–23	0.5	25 ± 5
VI(181–219d)	11.67	6	100	120	250	28 ± 2	0.5	25 ± 5
VII(220–238d)	10.49	6.67	100	120	250	28 ± 2	0.5	25 ± 5
VIII(239–278d)	10	7	100	120	250	28 ± 2	0.5	40 ± 5
IX(279–318d)	11.67	6	100	120	250	28 ± 2	0.5	40 ± 5
X(319–372d)	12.72	5.5	100	120	250	28 ± 2	0.75	40 ± 5
XI(373–411d)	14	5	100	120	250	28 ± 2	1.0	40 ± 5

The quantitative results showed that four-step reaction effectively eliminated the effect of primer dimmer (Zhang et al., 2004).

3. Results and discussion

3.1. Dynamics of nitrite accumulation resulted from nitritation and nitrification

Dynamics of NO_2^--N, NO_3^--N concentration and nitrite accumulation ratio (NAR) in the aerobic zone of MUCT reactor throughout operational period are shown in Fig. 1. In this study, combining low DO concentration with short hydraulic retention time (HRT) was the key method to improve nitrite accumulation and achieve nitritation. Previous studies verified that AOB have a stronger affinity with DO than NOB, and DO concentration of 0.5–1.0 mg/L is generally considered to favor nitritation establishment (Fux et al., 2006; Zeng et al., 2010). Thus, DO concentration was controlled at 0.5 mg/L most of the experimental time. Based on low DO (0.5 mg/L) operation in phase I, the HRT of phase II was decreased from 10.5 h to 8 h. Although nitritation and nitratation are simultaneous processes, the short HRT is favorable for the growth and enrichment of AOB, and thus NAR gradually increased. In phase III nitritation was established and maintained for 35 d with an average NAR of about 60%. Whereafter, wastewater temperature dropped to 18–23 °C due to seasonal changes in phase IV, leading to NAR decreasing. Even though HRT was further reduced to 6 h, NAR did not rise and the system was transformed to complete nitrification in phase V. High temperature is one of the key factors to establish nitritation. The SHARON process is a successful full-scale application of nitritation/denitritation at high temperatures of 30–35 °C and SRT of 1.5 d (Hellinga et al., 1998). Yet in the temperature range of 10–20 °C, high nitrite build-up can hardly be maintained due to the specific growth rate of NOB higher than that of AOB. From phase VI, since wastewater temperature gradually rose coupled with short HRT of 6–7 h and low DO concentration of 0.5 mg/L, NAR ascended again and the system entered into nitritation state. In phases VII, VIII, IX and X, nitritation was stably performed for total 153 d with an average NAR of 95%. In phase XI, DO concentration was increased to 1.0 mg/L to improve ammonia oxidation, resulting in NAR descent.

3.2. Effect of nitrite accumulation from nitritation on EBPR performance

The performance of EBPR, especially anoxic P removal in the MUCT reactor is shown in Fig. 2. Fig. 3 presents the variations of PO_4^{3-}-P concentration and PHAs along the reactor under nitritation and complete nitrification state. The average anoxic P uptake accounted for 88% of total P removal, indicating P was mainly removed by denitrifying P removal in anoxic zone. Electron acceptor is a key factor affecting the community structure of PAOs. Thus, the population structure of "*Candidatus* Accumulibacter" in this denitrifying P removal reactor will be different from that in a traditional EBPR reactor using oxygen as electron acceptor. Due to transformation of nitrification and nitritation, the electron acceptors recycled to the anoxic zone for denitrifying P removal were varying, i.e. nitrite and nitrate as electron acceptor during nitritation and nitrification period, respectively. Therefore, this study focused on the effect of nitrite and nitrate from nitritation and nitrification as electron acceptor on the population structure of "*Candidatus* Accumulibacter".

As shown in Figs. 2(a) and Fig. 3, the average anoxic P uptake accounted for 88% of total P removal under both nitritation and complete nitrification. That was resulted from the configuration of MUCT process. Since raw wastewater directly enters into anaerobic zone, "*Candidatus* Accumulibacter" can fully utilize the VFA in wastewater to form PHAs coupled with P-release. The VFA concentrations in the municipal wastewater varied in a range of 10.5–45.3 mg/L with an average of 23.6 mg/L. The VFA was almost exhausted in the anaerobic zone, coupled with PHAs up to the maximum (Fig. 3). Then the mixture rich in phosphorus enters into anoxic zone, where a certain concentration of NO_2^--N or NO_3^--N is present and carbon source is insufficient, providing a good condition for denitrifying P removal. Meanwhile, the PHAs content obviously decreased, which was oxidized to generate energy for P uptake and denitrification. As shown in Fig. 2(b), the average total P removal efficiency in each phase was 46.4%, 52.9%, 87.8%, 82.1%, 58.8%, 82.1%, 86.7%, 95.3%, 94.7%, 97% and 94.7%, respectively, positively correlated with the variations of nitrite accumulation ratio. The average P removal efficiency in phases III and VII ~ X with nitritation was higher by 40% than that in phases I and V with complete nitrification. The outcomes suggested that nitritation was advantageous for P removal from carbon-limited wastewater. That is possibly due to the following two reasons. Firstly, nitritation was performed in phases III and VII ~ X, and thus denitrifying P removal was mainly by nitrite pathway. Under complete nitrification state, about 5 mg/L of nitrate was detected in anoxic zone 1, and it would entered into anaerobic zone by anoxic recycle R_2. Whereas under nitritation state, no nitrate or nitrite was detected in anoxic zone 1. Mean C/N ratio of tested municipal wastewater was only 3, which hardly satisfied the demand of carbon source for both denitrification and P removal. Due to limiting carbon sources in raw wastewater, PHAs formed in anaerobic zone were likely

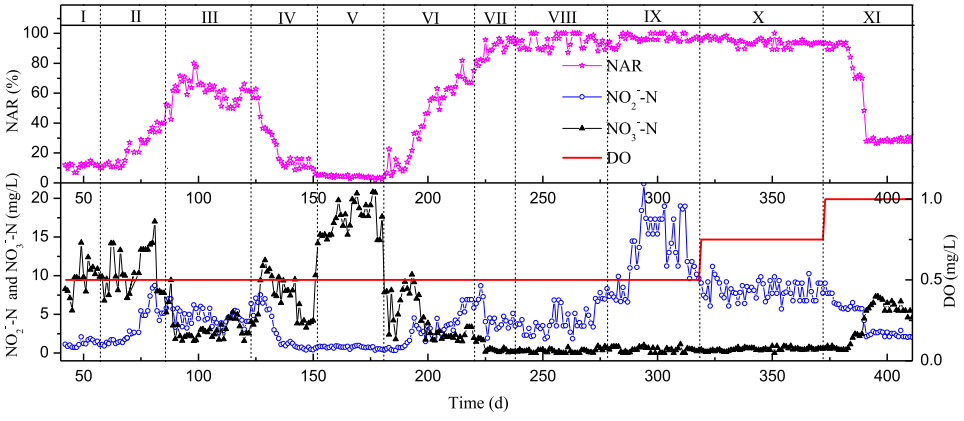

Fig. 1. Variations of NO_2^--N, NO_3^--N concentrations and nitrite accumulation ratios (NAR) in the aerobic zone.

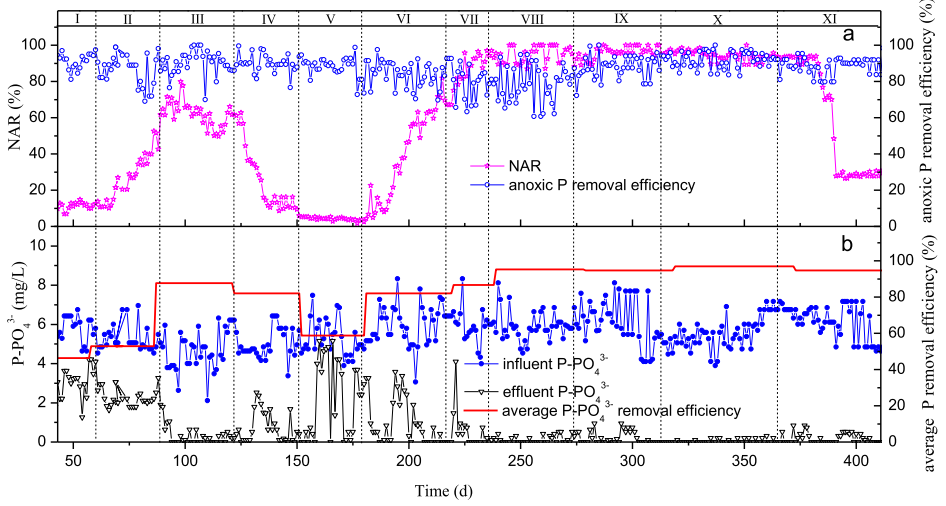

Fig. 2. Phosphorus removal in MUCT process.

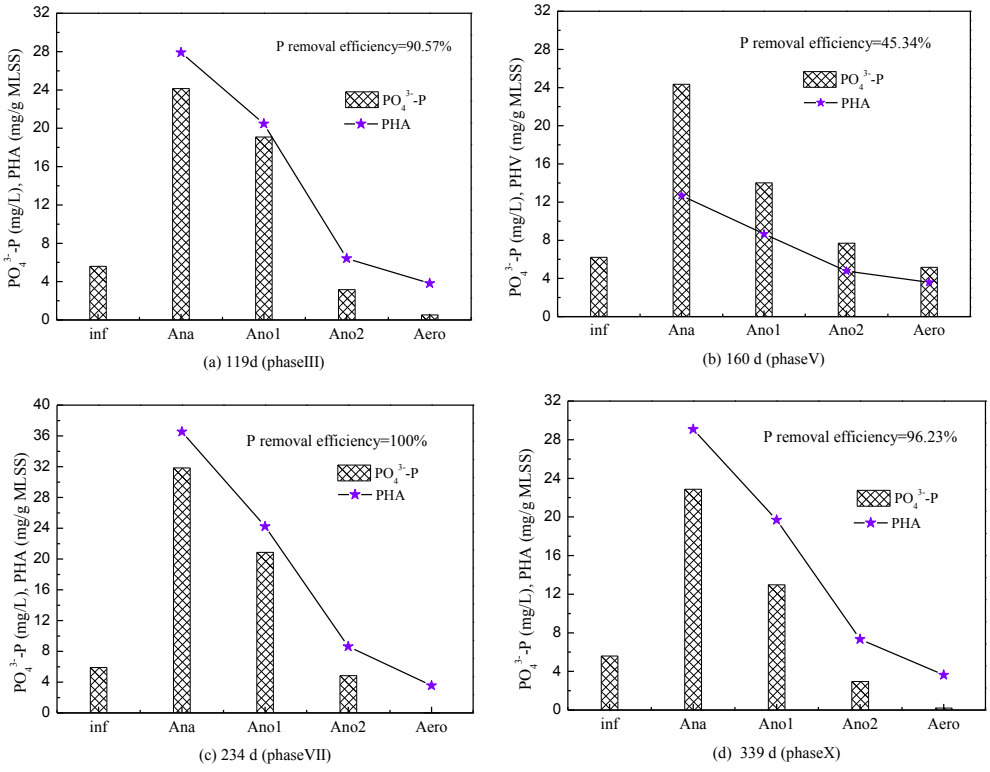

Fig. 3. Variations of PO_4^{3-}-P and PHAs along the reactor in MUCT process at the different experimental stages.

limiting. In the case of complete nitrification, limiting PHAs and carbon sources in raw wastewater resulted in incomplete denitrification. Therefore, return of nitrate to the anaerobic zone led to further consumption of carbon sources and decrease of PHAs formation. As shown in Fig. 3(b), the stored PHAs (12.7 mg/g MLSS) in anaerobic zone under complete nitrification was much lower than those (18–23 mg/g MLSS) under nitritation. While under nitritation state, PHAs was enough for nitrite reduction. Secondly, transformation between nitritation and complete nitrification led to varying of electron acceptor (nitrite or nitrate). Since "*Candidatus* Accumulibacter" has different capability to reduce nitrite or nitrate, denitrifying P removal possibly fluctuated under different nitrifying types.

3.3. Relevance of clade-level population dynamics of "Candidatus *Accumulibacter" to the performance of EBPR*

FISH based on 16S rRNA-targeted oligonucleotide probes was used to quantify the relative abundance of PAOs to total eubacteria, and compare with the results of quantitative PCR based on *ppk*1-specific primers. The proportions of PAOs to eubacteria in five sludge samples (on day 119, 131, 160, 255 and 339) were in a range of 1.5%~7.3% by FISH analysis. Yet 16S rRNA-targeted probes cannot reveal fine-scale differences within "*Candidatus* Accumulibacter" due to its highly conservative mechanism (He and McMahon, 2011a). Thus quantitative PCR based on *ppk*1-specific primers was also used in this study. The amplification efficiencies of standard

curves of five "*Candidatus* Accumulibacter" clades were from 90% to 105%, and the correlation coefficients were higher than 0.99, ensuring the precise quantification. The cell numbers of "*Candidatus* Accumulibacter" and bacteria, and "*Candidatus* Accumulibacter" percentages were calculated based on an assumption that "*Candidatus* Accumulibacter" contain 1 copy of *ppk*1 and 2 copies of 16S rRNA gene, and bacteria contain 4.1 copies of 16S rRNA (Martin et al., 2006; He et al., 2007). The sum of the *ppk*1 abundances of the five "*Candidatus* Accumulibacter" clades was consistent with the quantitative results using 16S rRNA primers, suggesting that the five clades can represent the total "*Candidatus* Accumulibacter" lineage in this system.

Fig. 4 presents the fractional abundance of the total "*Candidatus* Accumulibacter" lineage relative to the bacterial community (defined as FAB) under different NAR and P removal efficiency by quantitative PCR. The FAB from quantitative PCR was about 1%~6%, which was close to the results of FISH analysis.

As shown in Fig. 4, the P removal efficiency was strongly related with NAR, and FAB had a clearly positive correlation with NO_2^--N concentration, indicating "*Candidatus* Accumulibacter" primarily using nitrite as electron acceptor. During complete nitrification period (phases I and V) with NAR below 10%, NO_2^--N concentration was less than 1 mg/L, and the FAB was very low associated with poor performance of EBPR. On day 160 of phase V, the P removal efficiency was only 22%. After phase V, with nitrite increasing, the nitrate concentrations decreased to below 1 mg/L and NAR was higher than 90%. During this period the FAB rose with the increase of nitrite concentration, along with the improvement of P removal. On day 255 of phase VIII, the FAB reached 3.02%. When both nitrate and nitrite was simultaneously present, such as phases II, III and IV, the FAB had the same varying trend with nitrite concentration. During phases VII, VIII, IX and X with average NAR above 94%, the average P removal efficiency was as high as 96.5%, and was not affected by FAB variations. Therefore, we assumed that NO_2^--N concentration may determine the relative abundance of "*Candidatus* Accumulibacter", and the NAR likely determined "*Candidatus* Accumulibacter" bioactivity and function. Those are possibly due to the following two reasons: (1) P removal was mainly completed by denitrifying P removal in anoxic zone, and thus the concentrations of nitrite or nitrate as electron acceptor largely affected "*Candidatus* Accumulibacter" abundance, and (2) since organic carbon source in municipal wastewater with low C/N ratio was a key factor restricting P removal, denitrifying P removal using nitrite as primary electron acceptor during nitritation effectively utilized carbon source in raw wastewater, and thus improved "*Candidatus* Accumulibacter" bioactivity and performance of EBPR. The NO_2^--N concentration was 4.16 mg/L, 6.84 mg/L, 17.3 mg/L and 8.6 mg/L corresponding to day 234 of phase VII, day 255 of phase VIII, day 302 of phase IX and day 339 of phase X with an average NAR above 94%, respectively. From day 234 to day 255, FAB ascended with the increase of nitrite concentration, and "*Candidatus* Accumulibacter" abundance rose from 1.4×10^7 copies/(gVSS) to 2.0×10^7copies/(gVSS). However, on day 302 of phase IX, nitrite of high level of 17.3 mg/L obviously caused inhibition on "*Candidatus* Accumulibacter", and FAB decreased to 1.04%. Yet on this day, the P removal efficiency was still high, thereafter a decline on P removal was observed. That indicated the occurrence of nitrite inhibition followed by decline of P removal.

Table 2 shows the fractional abundance of each "*Candidatus* Accumulibacter" clade relative to the total "*Candidatus* Accumulibacter" in nine sludge samples. According to the proportion of each clade to total "*Candidatus* Accumulibacter", type II was much higher than type I. Previous studies indicated that "*Candidatus* Accumulibacter" from WWTPs was primarily affiliated with type II (McMahon et al., 2007; He et al., 2008), and type II could use nitrite rather than nitrate as electron acceptor (Flowers et al., 2009). In this study, real municipal wastewater and continuous flow process was used, similar to WWTPs. Moreover, during the experimental period of 369 d, nitritation had been operated for 304 d with high nitrite accumulating above 50%, namely a stable nitritation was achieved. Nitrite from nitritation was recycled into anoxic zone and could be used as electron acceptor by type II. Based on the two reasons, type II became the dominant "*Candidatus* Accumulibacter" varying from 90% to 100%, much more than type I. Among type II, clade IID was the most dominant clade (>90%) of "*Candidatus* Accumulibacter". Compared with clade IID, clades IIA, IIB and IIC were negligible (<4%). Type I was verified to be able to couple nitrate reduction with P-uptake in previous study (Flowers et al., 2009). Since complete nitrification was performed in phases I, V and XI, nitrate from nitrification could enter into anoxic zone and be used as electron acceptor by type I. As shown in Table 2, besides clade IID, clade I was present and became the secondly dominant "*Candidatus* Accumulibacter". On day 160 of phase V, NO_3^--N concentration was the highest (17.44 mg/L) and nitrite concentration was almost zero. Meanwhile, the proportion of clade I reached the maximum (5.08%) throughout the experimental period. Thereafter, the proportion of clade I descended with decrease of nitrate concentration. After phase VI (234 d), along with nitrate concentration below 1 mg/L, the clade I proportion further reduced, and almost disappeared in phase IX. Therefore, we assumed that clade I used nitrate as electron acceptor for denitrifying P removal, which was consistent with previous studies

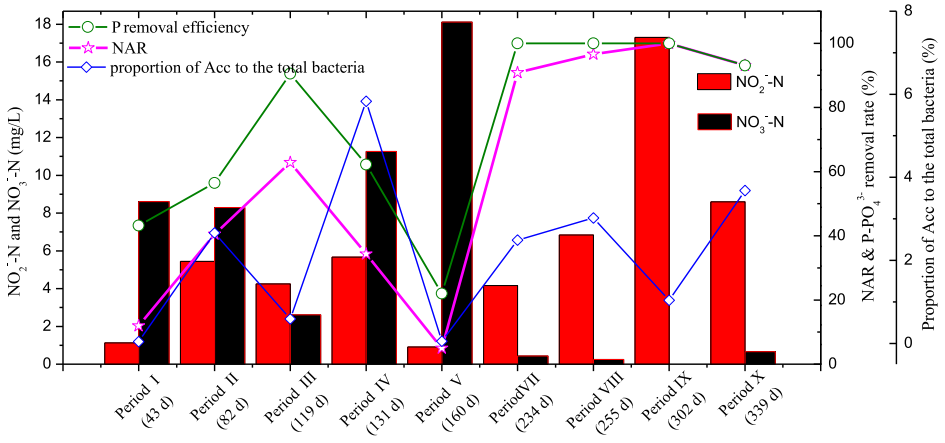

Fig. 4. Proportion of "*Candidatus* Accumulibacter" to total bacteria under different P removal efficiencies and nitrite accumulation ratios.

Table 2
Relative distribution of indicated clade in "*Candidatus* Accumulibacter".

Samples	Proportion of each clade to total accumulibacter (%)					NO_2^--N (mg/L)	NO_3^--N (mg/L)
	I	IIA	IIB	IIC	IID		
Day 43 (period I)	2.98	1.90	0.58	0.04	94.50	1.13	8.29
Day 82 (period II)	0.14	0.27	0.02	0.25	99.32	5.44	8.00
Day 119 (period III)	2.00	2.28	0.68	0.02	95.02	4.25	2.52
Day 131 (period IV)	0.02	0.06	0.00	0.04	99.88	5.67	10.85
Day 160 (period V)	5.08	1.54	0.38	0.03	92.97	0.91	17.44
Day 234 (period VII)	0.34	0.44	0.25	0.29	98.68	4.16	0.42
Day 255 (period VIII)	0.20	4.20	2.10	4.00	89.50	6.84	0.24
Day 302 (period IX)	0.05	0.36	0.07	0.09	99.43	17.31	0
Day 339 (period X)	0.03	0.10	0.08	0.17	99.62	8.60	0.64

(Carvalho et al., 2007; Flowers et al., 2009). Presence of nitrite was disadvantageous for the growth of clade I.

Abundance of each clade of type II in the nine sludge samples is given in Fig. 5. The abundance of each clade was strongly associated with nitrate and nitrite concentrations. Previous study found clade IIA could not couple nitrate reduction with P-uptake (Flowers et al., 2009), which suggested that clade IIA was able to use nitrite rather than nitrate as electron acceptor. As shown in Fig. 5(a), the abundance of clade IIA exhibited a clearly positive correlation with the nitrite concentration, and was insensitive to nitrate. On day 302 with the highest nitrite level of 17.31 mg/L, clades I, IIB and IIC almost disappeared. Although nitrites of 17.31 mg/L also made clade IIA abundance dramatically reduce, declining range was the smallest among all clades, indicating the clade IIA had a high tolerance to nitrite. Fig. 5(b) presents that clade IIB abundance was very low when nitrate was present, and it ascended with the increase of nitrite concentration when almost only nitrite existed. Therefore, clade IIB primarily used nitrite as electron acceptor, and was sensitive to nitrate. Presence of nitrate was disadvantageous for the growth of clade IIB. Fig. 5(c) shows that clade IIC was positively correlated with nitrite concentration, suggesting clade IIC was able to use nitrite as electron acceptor. As shown in Fig. 5(d) and Table 2, the proportion of clade IID to total Accumulibacter was the highest (above 92%), and the abundance of clade IID were strongly correlated with the nitrite concentrations. Thus, clade IID could use nitrite as electron acceptor, and played an important role for P removal in this study. Since the MUCT reactor had been operated for a long period under nitritation state, high level of nitrite accumulation from nitritation resulted in clade IID able to use nitrite as electron acceptor dominant, ensuring stable performance of denitrifying P removal. To our knowledge, this is the first report regarding the clade IID as the dominant denitrifying P removal

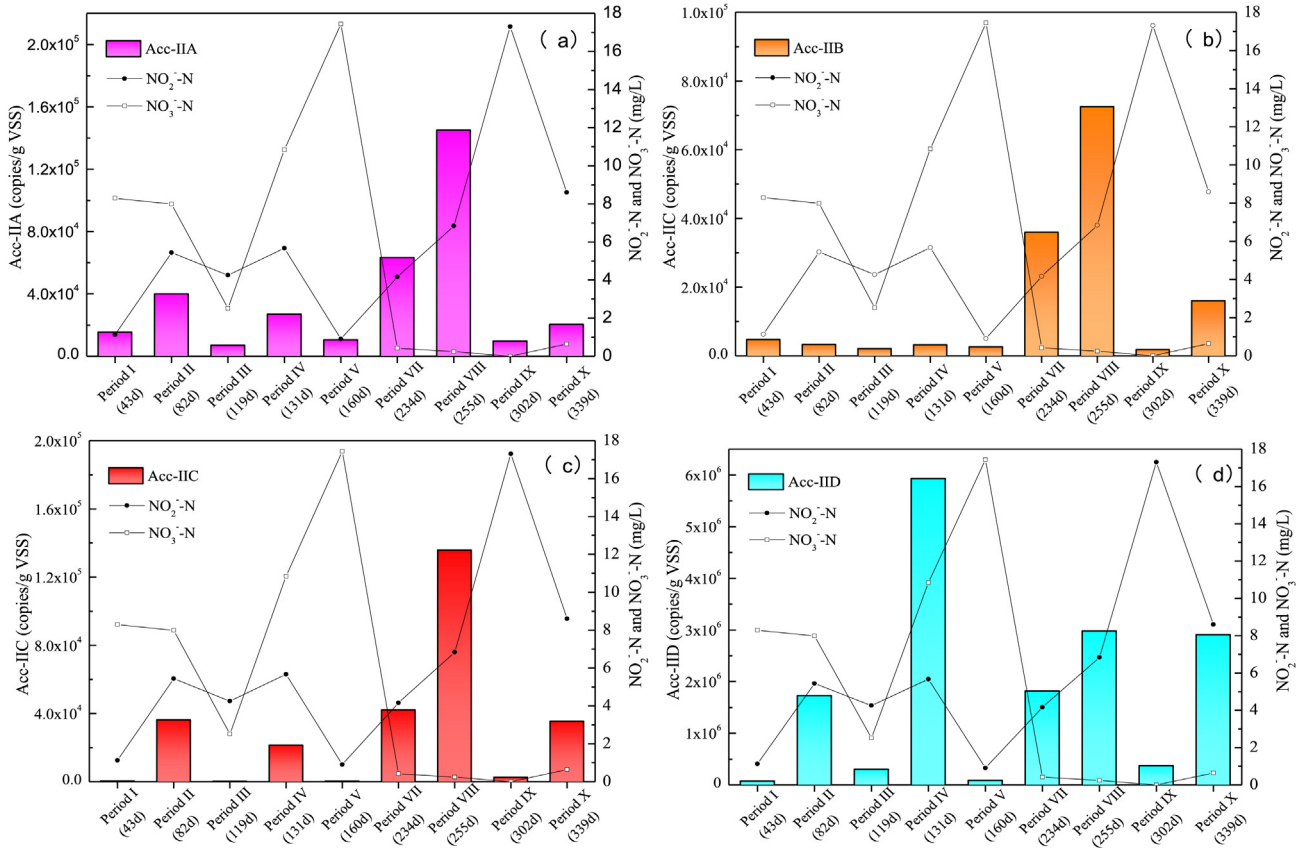

Fig. 5. Variation of each clade abundance of Type II.

bacteria in real municipal treatment system. Furthermore, poor performance of EBPR under complete nitrification state was likely resulted from clade IID as the dominant "*Candidatus* Accumulibacter" unable to couple nitrate reduction with P uptake. Fig. 5 also suggested that nitrite inhibition on all clades occurred when nitrite accumulation reached a high level (17 mg/L).

4. Conclusion

Nitritation and denitrifying P removal via nitrite pathway could be achieved by controlling DO concentration, hydraulic retention time and wastewater temperature. Real-time quantitative PCR method using *ppk*1 as a genetic marker could reveal the relevance of clade-level population dynamics of "*Candidatus* Accumulibacter" to the performance of EBPR. Clade IID using nitrite as an electron acceptor was always the dominant denitrifying P bacteria in the system, ensuring the stable performance of denitrifying P removal via nitrite pathway. Very few clade I (below 5%) using nitrate as electron acceptor were present in the reactor under complete nitrification, and then gradually disappeared during nitritation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.08.064.

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